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Optimization of in vitro Regeneration of Cowpea (*Vigna unguiculata* L.) using Computational Models

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Cowpea (*Vigna unguiculata* L.) is the most preferable cultivated crop in the World and can grow on poor soil with water scarcity. More than 80.0% of its cultivation is confined to the Sub-Saharan African continent. However, it is now one of the most preferable crops in Asia and Europe due to its low nutritional and water demand during cultivation under field conditions. In this study, the Bodrum Red cultivar of cowpea was used for in vitro regeneration. The 9-0 days old half cotyledonary node explant was used and placed on different concentrations of Benzylaminopurine (BAP), Indole-butyric acid (IBA), and Naphthalene acetic acid (NAA). The results were analyzed by ANOVA and response surface methodology (RSM). The ANOVA analysis revealed the maximum shoots from the medium enriched with 1.0 mg/L BAP with shot counts ranging from 1.17-3.11. The results were further analyzed by surface regression analysis which revealed the significant impact of NAA on callus formation. The results were also analyzed through Pareto charts and normal plots which confirmed the significant role of BAP and NAA on in vitro regeneration. The use of a response optimizer revealed the maximum shoot counts of 3.0 per explant from the medium supplemented with BAP. Results revealed the significance of RSM for optimizing input factors precisely for multiple input factors simultaneously. Advanced biotechnological approaches like genetic engineering and marker-assisted selection could enhance cowpea resilience to environmental conditions, ensuring its sustainability and adaptability across diverse agroecological zones with the aid of RSM.

Keywords: Tukey's B test, Pareto charts analysis, normal plot analysis, breeding programs, response surface methodology.

INTRODUCTION

The cowpea (*Vigna unguiculata* (L.) is an essential food legume that grows in tropical and subtropical climates worldwide. in various regions, particularly in Africa, Asia, Central America, and South America, it is a staple and multipurpose food legume consumed with grains, tender leaves, and fresh pods (Alemu *et al.,* 2016; Iftikhar *et al.,* 2021). Cowpea produces feed, fodder, hay, and silage for livestock, as well as green manure and cover crops to sustain soil productivity (Alemu *et al.,* 2016). In the agricultural system, it compensates for the loss of nitrogen absorbed by cereals, which improves soil quality. This is related to its phenomenal capacity to fix atmospheric nitrogen while performing well even on poor soils (Belay *et al.,* 2017). The crop also has the potential to inhibit weeds. As a drought-tolerant and warm-weather crop, it is a promising food and forage species in a typical tropical lowland climate (Bilatu *et al.,* 2012). This adaptable crop is a mainstay in the diets of millions of people because it improves nutritional intake and food security (Jayathilake *et al.,* 2018). Cowpeas are known for their rich nutritional composition; they are an excellent source of protein, carbs, and other nutrients important to human health (Jayathilake *et al.,* 2018). The benefits of cowpeas on diabetes, hyperlipidemia, and hypertension have all been studied for possible health advantages (Jayathilake *et al.,* 2018). Despite its nutritional value, cowpea has historically received less research attention compared to other cash crops, such as soybeans (Shiratori *et al.,* 2020).

The genetic diversity and breeding programs of cowpeas have been extensively studied to enhance their improvement and productivity (Aliyu *et al.,* 2023). Understanding the genetic architecture and gene pools of cowpeas is essential for developing improved varieties that can withstand various production constraints and environmental challenges (Huynh *et al.,* 2013). Genomic resources and genotyping platforms have been employed to boost the genetic potential of cowpea and other legume crops for improved food security (Salgotra

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and Stewart, 2022). These advancements in genomics have facilitated the use of molecular breeding approaches to optimize the nutritional quality and yield of cowpeas (Salgotra and Stewart, 2022**).** However, these objectives can be achieved by adopting biotechnological tools, and for this reason, optimization of in vitro regeneration protocol is highly essential. In modern days, optimization of protocols can be done by employing modern computation and machine learning models.

The Design of Experiments (DOE) is important in the field of plant sciences because it provides researchers with a systematic approach to decoding all aspects of plant biology, ecology, and agronomy (Timmusk and Behers, 2017). The design of Experiment provides a structured framework for designing, carrying out, and analyzing investigations of the factors that influence plant growth, development, and responses to environmental stimulation. Researchers can better understand plant physiology and adaptation by using advanced DOE references such as factorial designs, response surface methodology, and Taguchi methods (Swain *et al.*, 2021). The response surface methodology (RSM) is based on an advanced statistical technique and mathematics. The functional link between response variables and independent variables—one or more influencing factors—is studied using this method. Graphical techniques show the functional link and additional visual analysis that can be used to identify the optimum conditions or locations (Qadir *et al.*, 2019). This study aims to optimize in vitro regeneration methods using Response Surface Methodology (RSM) for the Bodrum Red cultivar of cowpea. Specifically, it evaluates the effects of concentrations of benzyl amino purine (BAP), indole-butyric acid (IBA), and naphthalene acetic acid (NAA) on callus induction and shoot formation. Furthermore, the study intends to evaluate the role of RSM in accurately optimizing various input components for improved in vitro regeneration protocol of cowpeas.

MATERIALS AND METHODS

***Plant Materials*:** Bodrum Red variety of cowpea registered for commercial production in Turkey was selected for in vitro regeneration studies. The seeds were first sterilized with 70% commercial bleach (3.5% NaOCl w/v) for 20 minutes. Then they were rinsed three times with distilled and sterilized

Table 1. ANOVA analysis of in vitro regeneration of Bodrum Cv. of cowpea.

Run No.	BAP (mg/L)	IBA (mg/L)	NAA (mg/L)	Mean	S.D.	95% CI	Mean	S.D.	95% CI
				**Callus Formation (%)			**Fresh Callus wt (mg)		
1	0.25	0	0	95.00^{AB}	10.00	(85.82; 104.18)	32.93C	6.21	(23.92; 41.94)
2	0.50	0	0	93.33^{B}	11.55	(82.73; 103.94)	30.00C	6.00	(19.59; 40.41)
3	1.00	0	0	86.67AB	11.55	(76.06; 97.27)	43.33BC	8.08	(32.93; 53.74)
4	0.25	0.25	0	53.33CD	11.55	(42.73; 63.94)	33.75C	7.58	(23.35; 44.16)
5	0.50	0.25	0	46.67D	11.55	(36.06; 57.27)	36.33C	15.50	(25.93; 46.74)
6	1.00	0.25	0	73.33BC	11.55	(62.73; 83.94)	81.33A	12.06	(70.93; 91.74)
7	0.25	0	0.25	100.0A	0.0	(89.4; 110.6)	84.79A	6.63	(74.38; 95.19)
8	0.50	0	0.25	100.0A	0.0	(89.4; 110.6)	64.67AB	13.61	(54.26; 75.07)
9	1.00	0	0.25	100.0A	0.0	(89.4; 110.6)	50.00BC	2.00	(39.59; 60.41)
10	0.25	0.25	0.25	100.0A	0.0	(89.4; 110.6)	50.67BC	6.11	(40.26; 61.07)
11	0.50	0.25	0.25	100.0AB	0.0	(87.0; 113.0)	33.00C	7.07	(20.25; 45.75)
12	1.00	0.25	0.25	86.67AB	11.55	(76.06; 97.27)	39.33BC	4.16	(28.93; 49.74)
				**Shoot Formation (%)			**Shoots per explant		
1	0.25	0	0	45.00A	10.00	(38.23; 51.77)	2.708A	0.672	(2.256; 3.159)
2	0.50	0	0	40.00B	0.00	(32.18; 47.82)	2.833A	0.577	(2.312; 3.355)
3	1.00	0	0	60.00A	0.00	(52.18; 67.82)	3.110A	0.191	(2.589; 3.631)
4	0.25	0.25	0	60.00A	0.00	(52.18; 67.82)	2.000AB	0.000	(1.479; 2.521)
5	0.50	0.25	0	40.00B	0.00	(32.18; 47.82)	2.000AB	0.000	(1.479; 2.521)
6	1.00	0.25	0	40.00B	0.00	(32.18; 47.82)	1.167B	0.289	(0.645; 1.688)
7	0.25	0	0.25	40.00B	0.00	(32.18; 47.82)	1.500B	0.500	(0.979; 2.021)
8	0.50	0	0.25	33.33B	11.55	(25.51; 41.15)	1.500B	0.500	(0.979; 2.021)
9	1.00	0	0.25	40.00B	0.00	(32.18; 47.82)	1.167B	0.289	(0.645; 1.688)
10	0.25	0.25	0.25	40.00B	0.00	(32.18; 47.82)	2.167AB	0.289	(1.645; 2.688)
11	0.50	0.25	0.25	30.0B	14.1	(20.4; 39.6)	2.500AB	0.707	(1.861; 3.139)
12	1.00	0.25	0.25	33.33B	11.55	(25.51; 41.15)	2.000AB	0.500	(1.479; 2.521)

**significant at *P0.001*

water, followed by culture in MS medium for germination. Germination started within 3-4 days but waited for 15 days to obtain cotyledonary node explants. The cotyledonary node explants were divided vertically in half and were used for in vitro regeneration studies (Aasim *et al.*, 2012).

The culture medium for germination and regeneration was prepared according to the standard procedure by adding MS (4.4 g/L), sugar (30 g/L), and agar (6.5 g/L). The medium was also supplemented with 1 g/L activated charcoal. The pH of the medium was adjusted after adding charcoal and before adding agar. The germination medium was prepared without adding any plant growth regulator (PGRs). Whereas in vitro regeneration medium was enriched with BAP, IBA, and NAA (Table 1). The medium was autoclaved at 121 ^{o}C and a pressure of 1.5 atm. After autoclaving, the 200 mg/L Sulcid antibiotic was also added to the culture medium for the reduction of bacterial contamination (Aasim *et al.*, 2012). All mediums for germination and regeneration were placed in the automated growth chamber with white light-emitting diodes (2030 LUX) for a 16h light photoperiod. The temperature and relative humidity of the growth chamber were adjusted at 24 ^{C} and 60.0% R.H.

For in vitro propagation, the explants were cultured in an MS medium containing different ratios of BAP, IBA, and NAA. After culturing the explants for 5 weeks, data were collected and analyzed by OneWay ANOVA analysis. The difference between the means was tabulated by Tukey's B test. Tukey's b is a correction factor used in Tukey's post hoc test to adjust for unequal sample sizes when comparing multiple treatment means in an analysis of variance (ANOVA). The results were further analyzed by Design of Experiment (DOE) by using response surface regression analysis of response Surface Methodology (RSM). The Pareto chart, normal plots, and response optimizer were also investigated. Both types of ANOVA and RSM analysis were performed using the Minitab 21.0 program. Furthermore, contour plots and surface plots were also used for optimization and constructed through the Design Expert 13.0 statistical program for better visual presentation.

RESULTS AND DISCUSSION

***ANOVA Analysis*:** The ANOVA analysis was performed for callus formation and callus weight, shoot formation, and number of shoots per explant (Table 1). As seen in Table 1, callus induction (%) was recorded between 46.67-100%, with the lowest callus induction obtained in the medium containing 0.50 mg/L BAP + 0.25 mg/L IBA. In general, lower callus formation was recorded in the medium containing IBA. Whereas the highest callus weights of 84.79 mg and 81.33 mg were obtained on medium containing 0.25 mg/L BAP+0.25 mg/L NAA and 1.00 mg/L BAP+0.25 mg/L IBA, respectively. In general, callus weight ranged between 30.00 and 84.79 mg (Table 1). ANOVA analysis of shoot formation was recorded between 30.0-60.0%. The highest shoot formation (60.0%) was obtained from the medium containing 1.0 mg/L BAP and 0.25 mg/L BAP + 0.25 mg/L IBA. When the results were examined for shoots per explant, it was noted that more shoots were obtained only in the media containing BAP without IBA or NAA. BAP+IBA-containing media generally gave more shoots than BAP-NAA-containing media. The number of shoots proved to be better in the media containing BAP-IBA-NAA. In general shoot counts ranged from 1.17-3.11. In vitro regeneration of legumes is dependent on multiple factors and the selection of appropriate explant) and plant growth regulators is highly significant (Kendir *et al.*, 2009; Aasim *et al*., 2011).

Surface Regression Analysis: The data obtained from the experiment were also analyzed by the surface regression model (Table 2). Surface regression analysis is a key of RSM which allows to analysis of the results from different dimensions and also optimizing multiple independent variables (up to 10) simultaneously with less number of combinations. The performance of the model was found significant at the p0.000 level (Aasim *et al.*, 2023). When the results of callus formation (%) were analyzed, the effects of BAP, BAP*IBA, and BAP*NAA ratios were not statistically significant, while IBA*NAA interaction was found significant (Hemmati *et al.*, 2020). When callus weight results were analyzed, NAA, BAP*IBA, BAP*NAA, and IBA*NAA showed statistically significant effects on callus weight. The analysis of shoot formation revealed that NAA and BAP*IBA were found statistically significant. When the results of the number of shoots per explant were analyzed, IBA*NAA showed a statistically significant effect, while all other inputs were found to be statistically insignificant (Singh *et al.*, 2012). The coded equations of all output parameters are given in Eq. 1-4.

Callus (%) = 92.2 - 11.9 BAP - 157.8 IBA + 82.2 NAA + 13.3 BAP*BAP + 41.9 BAP*IBA - 80.0 BAP*NAA + 462 IBA*NAA (Eq. 1)

Callus weight = 44.65 - 73.7 BAP - 30.0 IBA + 293.4 NAA + 75.2 BAP*BAP + 160.9 BAP*IBA - 283.8 BAP*NAA - 648.7 IBA*NAA (Eq. 2)

Regeneration (%) = 63.89 - 79.0 BAP + 53.3 IBA - 40.0 NAA + 71.1 BAP*BAP - 106.7 BAP*IBA - 7.6 BAP*NAA + 0.0 IBA*NAA (Eq. 3)

Shoots per explant = 2.767 + 1.00 BAP - 3.48 IBA - 6.48 NAA - 0.89 BAP*BAP - 2.54 BAP*IBA + 0.32 BAP*NAA + 32.28 IBA*NAA (Eq. 4)

***Pareto Charts and Normal Plot Analysis*:** The Pareto graphical results obtained by surface regression analysis are shown in Figure 1. Pareto charts are powerful tools that place the input factors in bars with the most significant factor at the top followed by the next factor and in this way the least significant factor at the bottom (Gani *et al.*, 2021). Any input factor that crosses the median line has a significant impact on the respective output parameter. The results showed that NAA

Table 2. Surface regression analysis of in vitro regeneration of Bodrum Cv. of cowpea

Source	DF	Adj SS	Adj MS	F-Value	P-Value	Adj SS	Adj MS	F-Value	P-Value
		Callus Formation (%)				Fresh Callus wt (mg)			
Model	7	10457.1	1493.88	13.95	0.000**	11798.7	1685.53	24.51	0.000**
Linear	3	7487.0	2495.66	23.31	0.000**	816.8	272.25	3.96	0.018
BAP	1	0.0	0.00	0.00	1.000	79.7	79.72	1.16	0.291
IBA	1	3010.7	3010.65	28.11	0.000**	61.5	61.54	0.89	0.352
NAA	1	4476.3	4476.32	41.80	0.000**	675.5	675.50	9.82	0.004**
Square	1	21.4	21.43	0.20	0.658	681.2	681.15	9.90	0.004**
BAP*BAP	1	21.4	21.43	0.20	0.658	681.2	681.15	9.90	0.004**
2-Way Interaction	3	2323.8	774.60	7.23	0.001**	9519.5	3173.16	46.13	0.000**
BAP*IBA	1	96.0	96.03	0.90	0.352	1416.3	1416.31	20.59	0.000**
BAP*NAA	1	350.0	350.00	3.27	0.081	4404.5	4404.49	64.04	0.000**
IBA*NAA	1	1877.8	1877.78	17.54	0.000**	3698.7	3698.67	53.77	0.000**
Error	28	2998.4	107.09	-	-	1925.9	68.78	-	-
Lack-of-Fit	4	1131.7	282.94	3.64	0.019	140.2	35.05	0.47	0.756
Pure Error	24	1866.7	77.78	-	-	1785.7	74.40	-	-
Total	35	13455.6	-	-	-	13724.6	-	-	-
		Shoot Formation (%)				Shoots per explant			
Model	7	2403.17	343.31	6.77	0.000**	13.5821	1.94030	11.05	0.000**
Linear	3	1272.18	424.06	8.37	0.000**	3.8811	1.29369	7.37	0.001**
BAP	1	66.67	66.67	1.32	0.261	0.5104	0.51042	2.91	0.099
IBA	1	98.25	98.25	1.94	0.175	0.5866	0.58656	3.34	0.078
NAA	1	1107.27	1107.27	21.85	0.000**	2.7841	2.78410	15.86	0.000**
Square	1	609.52	609.52	12.03	0.002**	0.0957	0.09571	0.55	0.466
BAP*BAP	1	609.52	609.52	12.03	0.002**	0.0957	0.09571	0.55	0.466
2-Way Interaction	3	625.40	208.47	4.11	0.015*	9.5188	3.17293	18.07	0.000**
BAP*IBA	1	622.22	622.22	12.28	0.002**	0.3526	0.35256	2.01	0.168
BAP*NAA	1	3.17	3.17	0.06	0.804	0.0055	0.00553	0.03	0.860
IBA*NAA	1	0.00	0.00	0.00	1.000	9.1607	9.16071	52.18	0.000**
Error	28	1419.05	50.68	-	-	4.9161	0.17557	-	-
Lack-of-Fit	4	352.38	88.10	1.98	0.129	0.8209	0.20522	1.20	0.335
Pure Error	24	1066.67	44.44	-	-	4.0952	0.17063	-	-
Total	35	3822.22	-	-	-	18.4982	-	-	-

**significant at *P0.001*

was the most effective for callus and shoot formation, BAP*NAA for callus weight, and IBA*NAA for shoot number. Investigation of individual parameters illustrated the order of C-B-BC-AC-AB-AA-A for callus formation, AC-BC-AB-AA-C-A-B for callus wt, C-AB-AA-B-A-AC-BC for shoot formation, and C-B-A-AB-AA-AC for shoots per explant.

The normal plots graphical results obtained by surface regression analysis are shown in Figure 2. Normal plots expressed the significant input factor as red square while blue circle means insignificant factor. Any input factor placed on the right side of the median line reflects the proportional relationship between input and output parameters (Xu *et al.,* 2019). Whereas the input factor on the left side shows the inverse proportional relationship between input and output parameters. When the results were analyzed, NAA exhibited a proportional effect on callus formation (Elias *et al.,* 2015). It means that callus formation can increase with the increase in NAA concentration. Whereas NAA harmed shoot formation and shoots per explant (Kaviani *et al.,* 2013). For callus weight, the BAP*IBA combination showed positive correlations on callus weight and a negative correlation for shoot formation (Tasiu *et al.,* 2021).

***Optimization using Response Optimizer*:** For the optimization of the results, the model was set at maximum for shoot formation and number of shoots per explant (Fakhrzad *et al.,* 2022). Whereas callus formation and callus weight were ignored (do not optimize). The solutions were adjusted for 5 different possible combinations and given in Table 3. When the results were examined, it confirmed the results and

Table 3. Optimization of in vitro regeneration of Bodrum cv. of cowpea using response optimizer

Solution	BAP (mg/L)	IBA (mg/L)	NAA (mg/L)	Shoots per explant	Shoot formation (%)	Composite Desirability
1	1.00	0.00	0.00	2.89	55.95	0.82
2	0.99	0.00	0.00	2.88	55.41	0.81
3	0.25	0.00	0.00	2.96	48.57	0.74
4	0.25	0.15	0.002	2.33	52.55	0.65
5	0.25	0.24	0.002	1.99	54.74	0.58

showed maximum shoot formation and shoot number by using only BAP at 1.0 mg/L closely followed by 0.99 mg/L BAP (Sinchana *et al.*, 2020). The results showed that 1.0 mg/L BAP is ideal for generation maximum shoot counts and shoot formation for Bodrum Cv. Response optimizer is considered a powerful tool for optimizing independent variables with predicted output (dependent) variables.

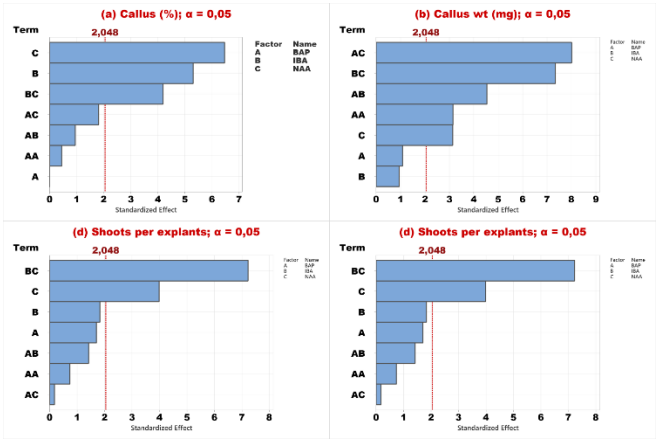

Figure 1. Pareto chart analysis of in vitro regeneration of Bodrum Cv. of cowpea.

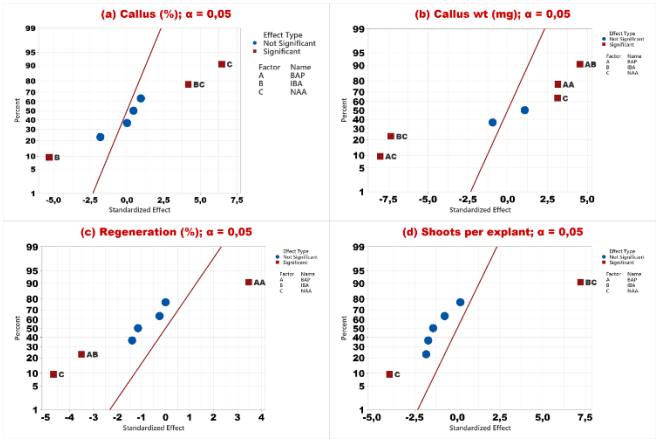

Figure 2. Normal plot analysis of in vitro regeneration of Bodrum Cv. of cowpea.

Conclusion: The study presents the successful in vitro regeneration of cowpea by using half cotyledonary nodes as explants, treated with various concentrations of BAP, IBA, and NAA. ANOVA analysis demonstrated that 1.0 mg/L BAP was effective in encouraging the production of shoots. Surface regression analysis demonstrated that NAA had a significant effect on the callus formation. The significance of BAP and NAA in in vitro regeneration was further supported by Pareto charts and normal plot analysis. Response optimizer further supported the results as maximum shoot counts with shoot formation can be achieved by using BAP alone at 1.0 mg/L. The study highlights the importance of response surface methodology in maximizing various input elements to achieve better results for in vitro regeneration. The results present promising prospects for developing cowpea production via enhanced in vitro regeneration methods which can be used for genetic modification, and sustainable farming approaches.

Authors contributions statement: Aasim M, Soomro SR, and Soomro SN did experiments, data curation, and article writing. Katırcı R. and Aasim M designed and analyzed the experimental data. Katırcı M reviewed and finalized the draft.

Conflict of interest: The authors declare no conflict of interest.

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***Ethical statement*:** This article does not contain any studies regarding humans or Animals.

***Availability of data and material*:** We declare that the submitted manuscript is our work, which has not been published before and is not currently being considered for publication elsewhere.

***Code availability*:** Not applicable.

Consent to participate: All authors participated in this research study.

***Consent for publication*:** All authors submitted consent to publish this research. article in JGIAS

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